

AD _____

Award Number: W81XWH-08-1-0572

TITLE: RNAi AS A ROUTE TOWARD BREAST CANCER THERAPY

PRINCIPAL INVESTIGATOR: GREGORY HANNON

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, NY 11724

REPORT DATE: September 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-09-2011		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 SEP 2010-31 AUG 2011	
4. TITLE AND SUBTITLE RNAi AS A ROUTE TOWARD BREAST CANCER THERAPY				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0572	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) GREGORY HANNON E-Mail: hannon@cshl.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT No abstract provided.					
15. SUBJECT TERMS RNAi, sequencing					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Page

Introduction.....	4
Body.....	4 - 8
Reportable Outcomes.....	8
Conclusion.....	8

Introduction

The goal of this innovator award is to continue to develop and apply RNAi-based screening methods to discover new routes toward breast cancer therapy. The project has three sets of goals. First is to integrate genomic and genetic information on available breast cancer cell lines to identify tumor-specific vulnerabilities and to understand genetic determinants of therapy resistance. Second is to probe the roles of breast cancer stem cells, with a particular emphasis on microRNAs. The third is to examine regions that determine familial susceptibility to breast cancer by applying novel, focal re-sequencing methods developed in the laboratory.

Body

Fourth-generation RNAi libraries

In collaboration with Scott Lowe and Steve Elledge, we developed a multiplexed validation assay for measuring shRNA potency, termed the “sensor assay.” We used this to generate approximately 250,000 measurements of shRNA efficacy, the largest such dataset ever generated. This provided sufficient information that we could devise a predictive algorithm, which we term shERWOOD, that can essentially predict the results of functional, sensor testing of shRNAs in silico. We are presently developing a web site that will make this tool available to the community, but we are also working toward the development of fourth-generation shRNA libraries based upon the approach.

All algorithms that predict effective RNAi tools tend to choose sequences that begin with a U. This is thought to have a structural basis in the interaction between the RNA and Argonaute, the key core of the RNAi effector complex. That 5' residue has been shown to reside in a binding pocket, which favors interaction with U. Therefore, the sequence space available for effective RNAi tools is really restricted to only 1/4 of the transcriptome. When the small RNA interacts with Argonaute, its 5' end is not available for pairing to the target RNA. Therefore, even though the 5' U contributes to RISC binding, it is irrelevant to target recognition. We therefore tested the idea that we could expand available sequence space by simply releasing the aforementioned constraint, in essence predicting on every position in the transcriptome and changing the small RNA guide that would pair to that site so that it contained a 5' U. This produced even higher scores in the algorithm and was especially important for small genes with limited numbers of potential target sequences.

We generated a collection of 1,320,000 oligonucleotides corresponding to shERWOOD shRNA predictions encompassing the human, mouse, rat, and fly genomes. These used both the conventional genomes and the 1U strategy. We confined our fourth generation libraries to REFSEQ genes and employed a series of heuristics to maximize the likelihood that our target sites would fall within constitutive exons. We have cloned these into our basic shRNA expression vector and begun to sequence verify and array the resulting libraries. Thus far, we have a unique collection of ~60,000 shRNAs for the human genome, and work on mouse and rat is ongoing. We have also signed a term

sheet with a distribution company to make the materials available and have begun to validate fourth generation shRNAs in a variety of rigorous assays.

Screening cell lines for new therapeutic targets

Over the past year, we have continued to work toward large scale screens of breast cancer cell lines. We have continued to have difficulty with drug sensitivity measurements provided by collaborating groups. However, we have managed to make substantial progress on in vitro screening. A table with the current status of this effort is presented below.

Breast Cancer Cell Lines	Screening Conditions	# of timepoints	Status
<i>Her2+ treatment category</i>			
JIMT1	straight lethal	T=11	Screen completed/sequenced
JIMT1	lapatinib IC20	T=11	Screen completed/sequenced
MDA-MB-453	straight lethal	T=5	Screen completed/sequenced
MDA-MB-453	lapatinib IC20	T=5	Screen completed/awaiting sequencing
MDA-MB-361	lapatinib IC80	T=4	Screen completed/awaiting sequencing
EFM192A-TR	straight lethal	T=4	Screen completed/awaiting sequencing
EFM192A-TR	trastuzumab (15ug/ml)	T=4	Screen completed/awaiting sequencing
HCC1954	straight lethal	T=4	Screen completed/microarray analysis completed
<i>ER+ treatment category</i>			
MCF-7 Parental + E2	straight lethal	T=4	Screen completed/awaiting sequencing
MCF-7 Parental + E2	without E2	T=4	Screen completed/awaiting sequencing
MCF-7 Parental + E2	without E2/ plus tamoxifen	T=3	Screen completed/awaiting sequencing
MCF-7-EDR	with E2	T=4	Screen completed/awaiting sequencing
MCF-7-EDR	without E2	T=4	Screen completed/awaiting sequencing
MCF-7-TAMR	with E2	T=4	Screen completed/awaiting sequencing
MCF-7-TAMR	without E2/ plus tamoxifen	T=4	Screen completed/awaiting sequencing
ZR-75-1 Parental + E2	straight lethal	T=4	Screen completed/awaiting sequencing
ZR-75-1 Parental + E2	without E2	T=4	Screen completed/awaiting sequencing
ZR-75-1 Parental + E2	without E2/plus tamoxifen	T=4	Screen completed/awaiting sequencing
ZR-75-1 -EDR	with E2	T=4	Screen completed/awaiting sequencing
ZR-75-1 -EDR	without E2	T=4	Screen completed/awaiting sequencing

ZR-75-1-TAMR	with E2	T=4	Screen completed/awaiting sequencing
ZR-75-1-TAMR	without E2/plus tamoxifen	T=4	Screen completed/awaiting sequencing
T47D	straight lethal	T=4	Screen completed/microarray analysis completed

TN/Basal treatment category

Hs578T	straight lethal	T=7	Screen completed/awaiting sequencing
MDAMB231	straight lethal	T=4	Screen completed/awaiting sequencing
MDAMB468	straight lethal	T=4	Screen completed/awaiting sequencing
MDAMB436	straight lethal	T=4	Screen completed/microarray analysis completed
HCC1143	straight lethal	T=4	Screen completed/microarray analysis completed
HCC1937	straight lethal	T=4	Screen completed/microarray analysis completed
SUM149	straight lethal	T=4	Screen completed/microarray analysis completed
SUM1315	straight lethal	T=4	Screen completed/microarray analysis completed

Normal cells

HMEC	straight lethal	T=4	Screen completed/microarray analysis completed
------	-----------------	-----	--

We have completed screens in JIMT-1 and MDA-MB436 to the point that they have been fully analyzed. This involved the development of custom analysis pipelines that use discrete sequencing counts rather than our more traditional microarray-derived datapoints. We immediately noted that, unlike screens of prior libraries, many of the genes that were scored as positive were being hit with multiple shRNAs. For example, in MDA-MB436, 3000 scoring shRNAs collapsed to only 1800 genes. These screens, which were carried out with the third-generation library, are therefore giving much more robust data than we have ever seen before.

We are currently in the process of completing analysis of the remaining screens listed about and designing methods to integrate the data to nominate candidate targets.

One explicit goal of the innovator expansion proposal was to identify pathways that are important in mammary stem cells. It has become increasingly clear that tumor cells, like normal cells, are driven by self-renewing compartments known as tumor-initiating or cancer stem cell populations. These cells exhibit higher resistance to targeted therapy than the rest of the tumor cell population. Thus it is important to understand the essential pathways that drive tumor-initiating cells and how these signatures differ from those that enable normal breast progenitor cells to survive. To mark progenitor cells in normal mouse mammary epithelial cells, we have applied an approach developed by E. Fuchs laboratory (Rockefeller University) to identify/purify label-retaining cells (LRCs) that mark the skin stem cell niche. The system is built upon the premise that stem cells

are slow cycling and active for the keratin5 promoter. It utilizes a tetracycline-responsive promoter driving histone 2B-GFP (H2B-GFP) in a transgenic mouse model expressing the tet repressor-VP16 (tTA) transgene from the K5 promoter. In the absence of doxycycline, the expression of GFP is high in epithelial cells. After feeding the animal doxycycline for a period of several weeks only very small populations of epithelial cells retain GFP fluorescence.

Using this system we have isolated LRCs from mammary epithelial cells and have demonstrated their self-renewal potential both *in vitro* (mammosphere formation assay) and *in vivo* (reconstitution of mammary gland). Furthermore, we have profiled the H2B-GFP+ cells for transcriptome, methylome, and miRNA expression analysis. Comparison of the transcriptomes of LRCs and other subpopulations of the mammary gland (luminal ductal cells, luminal alveolar cells, luminal progenitors, myoepithelial progenitors, and myoepithelial cells) has allowed us to identify new a cell surface-specific marker for the H2B-GFP+ cells, CD1. CD1-specific cell populations were also found to be present in human basal breast cancer cell lines. We are currently testing if these human CD1-specific tumor cell populations have self-renewing capacity.

To find essential genes/survival pathways for H2B-GFP+ breast cancer progenitor cells, we have made a focused shRNA library targeting the MaSC genes, which are highly expressed in the H2B-GFP+ compartment but not in other normal mammary cell types. This MaSC shRNA library was then used to perform a well-by-well RNAi screen to test the effect of each individual shRNA on the survival of COMMA-D cells (normal mouse mammary epithelial cell line) and 4T-1 cells (mouse mammary basal-like, metastatic cell line). Several candidate genes from this screen are being tested to determine whether they are essential for survival of human triple-negative breast cancer cells.

To explore the role of epigenetics in cancer cell survival, we have completed another well-by-well RNAi screen in 4T1 cells using a collection of 1,100 shRNAs targeting 243 genes involved in chromatin regulation. BRD4, a gene that was recently identified as a therapeutic target in acute myeloid leukemia (C. Vakoc, CSHL), was one of the top hits (three independent shRNAs were identified). We are now testing whether any of the breast cancer subtypes exhibit sensitivity towards BRD4 inhibition.

Finally, we identified an additional hit, BBTF, which is an epigenetic regulator upon which breast cancer lines seem selectively dependent (unlike BRD4, which scores as a hit in a number of different cancer types). BBTF is a bromo-domain protein which is likely to be amenable to the design of chemical inhibitors via strategies similar to those used for BRD4. We are presently validating BBTF as a hit across our human cell line panel and verifying that it is also essential when these cells form tumors *in vivo*. Once these studies are completed, we will search for partners in the design of drugs against this molecule.

Epigenetic characterization of the mammary epithelial lineage

Finally, we have undertaken a full epigenetic characterization of the mouse mammary epithelial lineage from nulliparous and parous mice. In part, our goal has been to understand how DNA methylation patterns change as cells differentiate along this lineage and to understand what discriminates stem cells from their more mature progeny. We hope to use this information in reference to similar profiles of breast cancer cells to learn something about the role of epigenetics in the process of tumor formation. A second key goal is to ask whether mammary cell types show epigenetic changes upon pregnancy. If so, our hope is that these will somehow explain the strong protective effect of early pregnancy against the development of breast cancers. Since this protection is essentially life-long, it is not difficult to imagine that some shift in the nature of mammary epithelial cell populations might underlie this observation.

Thus far, we have completed an analysis of hypomethylated regions in mammary epithelial cells isolated from virgin mice by conventional marker strategies and H2B label retaining cells (see above). All that is missing is the profiling of CD1-positive cells and data for this has been collected but not analyzed. We see highly specific methylation signatures that are individual to the stem cell and to each lineage and note both methylation gains and losses as cells differentiate.

We have also made substantial progress on the analysis of parous animals. Though this analysis is at its earliest stages, all indications are that each mammary cell type will show major shifts in methylation patterns. A key goal for the next year is to complete a manuscript reporting results of analysis of the virgin animals and to complete comparisons of virgin and parous mammary glands.

Reportable outcomes

A fourth generation human shRNA library comprising ~60,000 shRNAs targeting ~19,000 genes

Conclusions

We continue to make progress toward the major goals of this application. This year, some of our most important strides have been in understanding mammary epithelial biology and in the development of highly optimized shRNA tools.